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Title: Nitro-oxidative stress correlates with Se tolerance of *Astragalus* species

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Running title: Selenate-induced nitro-oxidative stress in *Astragalus* species

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ABSTRACT

At high concentrations selenium (Se) exerts phytotoxic effects in non-tolerant plant species partly due to the induction of nitro-oxidative stress; however, these processes are not fully understood. In order to get a more accurate view about the involvement of nitro-oxidative processes in plant Se sensitivity, this study aims to characterize and compare Se-triggered changes in reactive oxygen (ROS) and nitrogen species (RNS) metabolism and the consequent protein tyrosine nitration as a marker of nitrosative stress in non-accumulator *Astragalus membranaceus* and in Se hyperaccumulator *Astragalus bisulcatus*.

The observed parameters (Se accumulation, microelement homeostasis, tissue-level changes in the roots, germination, biomass production, root growth, cell viability) supported that *A. membranaceus* is Se sensitive while the hyperaccumulator *A. bisulcatus* tolerates high Se doses. We first revealed that in *A. membranaceus*, Se sensitivity coincides with the Se-induced disturbance of superoxide metabolism leading to its accumulation. Furthermore, Se increased the production or disturbed the metabolism of RNS (nitric oxide, peroxynitrite, S-nitrosoglutathione) consequently resulting in intensified protein tyrosine nitration in sensitive *A. membranaceus*. In the (hyper)tolerant and hyperaccumulator *A. bisulcatus*, Se-induced ROS/RNS accumulation and tyrosine nitration proved to be negligible suggesting that this species is able to prevent Se-induced nitro-oxidative stress.

Keywords: *Astragalus ssp; nitro-oxidative stress; reactive oxygen species; reactive nitrogen species; selenate*

Abbreviations: CAT, catalase; DAF-FM DA, 4-amino-5-methylamino- 2′,7′-difluorofluorescein diacetate; DHE, Dihydroethidium; DHR, dihydrorhodamine 123; DPI, diphenylene iodonium; EDTA, ethylene-diamine-tetraacetic acid; FDA, fluorescein diacetate;

GSNO, S-nitrosoglutathione; GSNOR, S-nitrosoglutathione reductase; H_2O_2, hydrogen peroxide; MES, morpholine-ethansulphonic acid; NBT, nitroblue tetrazolium; NBT/BCIP, nitroblue tetrazolium/ 5-Bromo-4-chloro-3-indolyl phosphate; NO, nitric oxide; $^{\cdot}NO_2$, nitrogen dioxide radical; N_2O_3, dinitrogen-trioxide; N_2O_4, dinitrogen tetroxide; NOX, NADPH oxidase; NR, nitrate reductase; $O_2^{\cdot-}$, superoxide; $OH^{\cdot}$, hydroxyl radical; $ONOO^-$, peroxynitrite; PODs, peroxidases; RNS, reactive nitrogen species; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; Se, selenium; SIN-1, 3-morpholino-sydnonimine; SMT, selenocysteine methyltransferase; SNO, S-nitrosothiol; SOD, superoxide dismutase.

INTRODUCTION

Selenium (Se) is a non-metal element which seems to be non-essential for higher plants. Still, its chemical similarity with sulphur (S) results in its uptake and metabolism *via* S transporters and pathways (Pilon-Smits and Quinn, 2010). Moreover, a few plant species not only take up but accumulate or hyperaccumulate high Se levels in their tissues.

The ability of Se hyperaccumulation has been described in 45 plant taxa in six families (White, 2016). The *Astragalus* (*Fabaceae*) genus is the most representative since large number of species (25) in the genus have the ability to take up and tolerate high concentrations of selenium (Shrift, 1969). Species like *Astragalus bisulcatus* grow on seleniferous soils and can accumulate over 1000 μg g^{-1} DW Se (up to 1% of its dry weight). Hyperaccumulators possess 10-100-fold higher endogenous Se content as well as higher Se:S ratio compared to non-accumulators (White *et al.*, 2007). Another distinctive feature of hyperaccumulators is the active sulphate/selenate assimilation which is suggested by the dominance of organic Se forms (gamma-glutamyl-methyl-selenocysteine) in their tissues. Hyperaccumulators can be characterized by notable root-to-shoot Se translocation (Mehdawi and Pilon-Smits, 2012). Species like *A. bisulcatus* are able to sequester Se in their epidermis and trichomes, which may have a role both in defence and in Se stress mitigation (Freeman *et al.*, 2006). The mechanism responsible for Se hyperaccumulation is the constitutive expression of several SULTR transporters, which contributes to the preferential uptake of selenate over sulphate (Cabannes *et al.*, 2011). Also, the expression of certain enzymes involved in selenate/sulphate assimilation is enhanced in hyperaccumulators resulting in greater inorganic-organic conversion (Freeman *et al.*, 2010). Moreover, hyperaccumulators express selenocysteine methyltransferase (SMT) which is responsible for the conversion of toxic selenocysteine to methyl-selenocysteine (Sors

et al., 2009). Selenium tolerance is also typical for hyperaccumulators; however, the molecular mechanism of this ability is only partly understood.

High tissue concentrations of inorganic selenium forms can induce the production of reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$) leading to oxidative stress (Van Hoewyk, 2013). The amount of the generated ROS and consequently the redox homeostasis is precisely controlled by antioxidant mechanisms. Beyond the enzymatic components like superoxide dismutase (SOD), catalase (CAT) and peroxidases (PODs), non-enzymatic antioxidants such as ascorbate and glutathione (GSH) play crucial role in the defence against oxidative damage (Das and Roychoudhury, 2014). For Se-induced ROS accumulation, GSH and its depletion seems to be responsible (Van Hoewyk, 2013). According to previous data, hyperaccumulators prefer to produce organic Se forms presumably in order to avoid oxidative stress (Freeman *et al.*, 2006, Van Hoewyk, 2013).

Besides ROS, reactive nitrogen species (RNS) are also formed as the effect of environmental stresses like Se exposure (reviewed by Kolbert *et al.*, 2016). This group of nitric oxide (NO)-related molecules consists of peroxynitrite ($ONOO^-$), S-nitrosoglutathione (GSNO), dinitrogen trioxide (N_2O_3), dinitrogen tetroxide (N_2O_4), nitrogen dioxide radical ($^{\cdot}NO_2$) (Corpas *et al.*, 2007). The overproduction of RNS leads to nitrosative stress during which one of the principle mechanism is the nitration of tyrosine residues in certain proteins yielding 3-nitrotyrosine (Corpas *et al.*, 2013a). This modification causes structural and functional changes in the affected proteins. In most published cases, tyrosine nitration results in activity loss of the target plant proteins (Kolbert *et al.*, 2017) or it can negatively affect signal transduction through the prevention of tyrosine phosphorylation (Galetskiy *et al.*, 2011). Selenium-induced increase in protein tyrosine nitration and in oxidative parameters (ROS levels, lipid peroxidation, antioxidants) has been revealed in the leaves of non-accumulator pea (Lehotai *et al.*, 2016). Also, the relationship between the toxicity of selenium forms and protein

tyrosine nitration has been evaluated in non-accumulator *Arabidopsis thaliana* and secondary accumulator *Brassica juncea* (Molnár *et al.*, 2018ab) but there is no knowledge about RNS metabolism and protein nitration in Se hyperaccumulator plants such as *A. bisulcatus*. Another species in the *Astragalus* genus is *Astragalus membranaceus,* which is considered to be pharmacologically relevant. The root of this *Astragalus* species has been used in Chinese medicine for thousands of years because of its general strengthening effect. Based on the literature, in modern medicine it can provide perspective for the prevention and therapy of cerebrovascular, cardiovascular, neurodegenerative and liver diseases (Yang *et al.*, 2013). Despite the significance of *A. membranaceus*, we know little about its Se accumulation and tolerance as well as about reactive species metabolism and nitrosative stress.

Therefore, this comparative study aims to explore the possible differences in selenium-modified ROS and RNS metabolism and the consequent protein tyrosine nitration using the hyperaccumulator *Astragalus bisulcatus* and *Astragalus membranaceus* as another species in the same genus. The better understanding of tolerance mechanisms of Se hyperaccumulator plant species is of particular significance in phytoremediation (Gupta and Gupta, 2017) and in biofortification (Wu *et al.*, 2015) as well as in ecological (Schiavon and Pilon-Smits, 2017) point of view. Furthermore, examination of Se accumulation and tolerance of the medicinal herb *A. membranaceus* can have importance in human health aspect.

RESULTS

Selenium uptake, accumulation and microelement imbalance

Selenate-induced selenium accumulation showed differences in the organs of *Astragalus* species (Fig 1). In the root tissues of *A. membranaceus*, Se concentration significantly enhanced as the effect of increasing exogenous selenate supplementation (Fig 1A). In case of *A. membranaceus* cotyledons, Se accumulation was not concentration-dependent and proved to be lower compared to the root (Fig 1B). The Se content measured in cotyledons of 50 or 100 μM selenate-treated *A. membranaceus*, did not reach the endogenous Se content of the control *A. bisulcatus*. The root of the hyperaccumulator *A. bisulcatus* showed moderate Se accumulation (Fig 1A), while in the cotyledons a remarkable, concentration-dependent increase of Se content was observed (Fig 1B). In case of 100 μM selenate supplementation, the accumulated Se exceeded 1700 $\mu g\ g^{-1}$ DW concentration in the cotyledons of *A. bisulcatus*. It has to be mentioned that significant difference was observed in the endogenous Se contents of untreated *Astragalus* plants. Cotyledons of *A. bisulcatus* contained 200-fold more selenium than the same organs of *A. membranaceus* (Fig 1B). Regarding the root, similar but much smaller (16-fold) difference was revealed (Fig 1A).

Selenate exposure led to the modification of microelement concentrations in the organs of *Astragalus* species (Table 1). Of the examined microelements, the contents of the essential Fe, Zn, Mn and B showed notable reduction especially in the cotyledons of *A. membranaceus*. However, the concentration of the above mentioned elements were not affected at all or just slightly changed by selenate in *A. bisulcatus* cotyledons. Regarding the root system, more serious effects were observed in case of *A. membranaceus* compared to *A. bisulcatus*. E.g. Fe concentration decreased by 30% in *A. membranaceus* but only by 15% in *A. bisulcatus*. Contrary to the other microelements, Mo concentrations in *A. membranaceus* organs

significantly increased as the effect of Se treatments. In *A. bisulcatus*, the concentrations of Mo were decreased or were not modified by selenium (Table 1).

Growth and Se tolerance of *Astragalus* species

Selenium tolerance of *Astragalus* species was evaluated by germination capacity, biomass production, root meristem viability and root elongation on selenate-supplemented medium.

Both species showed ~85% germination under control conditions and this good germination capability was retained by *A. bisulcatus* on 50- and 100 μM selenate-treated plates (Fig 2A). In contrast, the presence of selenate significantly and concentration-dependently reduced the germination percentage of *A. membranaceus*. In case of 100 μM Se treatment, 55% of *A. membranaceus* seeds placed on the medium were germinated, while *A. bisulcatus* showed better (~70%) germination performance.

With regard to biomass production, 14-days-old, untreated individuals of the species possessed similar shoot weight (Fig 2B). Although, the root fresh weight of control *A. membranaceus* was significantly smaller (Fig 2C) and the phenotype of the root system notably differed from that of *A. bisulcatus* (Fig 2D). Both concentrations of exogenous Se (50 and 100 μM) negatively affected shoot (40 and 46% reduction, respectively) and root growth (57 and 75% reduction, respectively) of *A. membranaceus* (Fig 2BC) and a brown discoloration was visible on the root surface of Se-treated plants (Fig 2D). In contrast, *A. bisulcatus* showed significantly slighter growth inhibition, since the root biomass was affected only by the highest Se dose (30% reduction) and none of the treatments inhibited shoot growth (Fig 2 BCD).

Selenium tolerance correlates with the capability of maintaining primary root (PR) elongation, therefore Se tolerance index can be calculated from PR length data (Tamaoki *et al.*, 2008). Compared to the 100% tolerance of the untreated plants (indicated by dashed line in Fig

3A), 50 or 100 μM selenate resulted in 35 or 25% tolerance index of *A. membranaceus*, respectively (Fig 3A). However, *A. bisulcatus* was able to maintain its root growth and Se even slightly increased elongation resulting in tolerance indexes around or above 100%. Furthermore, we examined the Se tolerance of the species by evaluating viability of the root meristem cells using fluorescein diacetate staining (Fig 3BC). As expected from the previous data, the meristem cells of *A. membranaceus* showed 50 or 85% viability loss as the effect of 50 or 100 μM selenate exposure, respectively. Even though root elongation of *A. bisulcatus* was negatively affected by none of the applied Se doses (Fig 3A), root meristem cells suffered 50% viability loss as the effect of the highest Se concentration (Fig 3BC). We acknowledge that the application of plant tissues with highly reduced viability might limit the reliability of the data. At the same time, the choice of the 14 days-long treatment period proved to be necessary for the appearance of the effect, as well as for the emergence of tolerance in this comparative *Astragalus* system (Suppl Fig 1).

Se-induced tissue-level changes in the roots

To evaluate the Se-induced tissue-level changes in the root structure of both *Astragalus* species, we measured the diameter of the root, the thickness of the cortex and the diameter of the vascular cylinder (stele). Both untreated and Se-treated *A. membranaceus* plants had thick roots and Se application did not significantly affect root diameter ($F= 1.25$, $p= 0.29$). In case of control and 50 μM Se-treated plants, *A. membranaceus* had nearly twice as thick roots as *A. bisulcatus* (Fig 4A). When 100 μM Se was added to the media, the roots of *A. bisulcatus* exhibited remarkable thickening which value was similar to that of *A. membranaceus*. This tendency was also confirmed by analysis of correlation ($r= 0.82$, $p< 0.001$). Similarly, the sensitive *A. membranaceus* had significantly thicker root cortex than the Se-hyperaccumulator *A. bisulcatus* in both control and 50 μM Se-treated plants, but it was almost the same in the

roots of 100 μM Se-treated plants of both species (Fig 4B). Increasing Se levels significantly enhanced the thickness of the cortex in the case of *A. bisulcatus* ($F= 403.88$, $p< 0.001$; $r= 0.88$, $p< 0.001$), while remarkable increase was found only in the root cortex of 50 μM Se-treated *A. membranaceus* ($F= 33.88$; $p< 0.001$; $r= 0.34$, $p<0.001$). There was a remarkable increase of stele diameter in *A. membranaceus* roots exposed to 50 μM Se, while it significantly decreased compared to control after 100 μM Se application. The size of the stele in the roots of *A. bisulcatus* was only affected by the highest Se stress (Fig 4C). The stele of control and 50 μM Se-treated *A. membranaceus* roots was at least twice thicker than that of *A. bisulcatus*. Selenium stress-induced deposition of callose was investigated in aniline blue (AB)-stained root sections taken from the mature zone. Significantly higher fluorescence was found in Se-treated roots of *A. membranaceus* compared to control, while it diminished after Se application in *A. bisulcatus* (Fig 4D). Lignin and suberin deposition was visualized using Auramine O staining in the root sections. An intense fluorescence was found in the stele in control roots of both species due to the xylem vessels (Fig 4E). In the Se-treated roots of *A. membranaceus* a slight fluorescence appeared on the surface (exodermis) of the roots. This staining exhibited both the endodermis and the exodermis in the roots of Se-treated *A. bisulcatus.*

Se-induced changes in ROS and RNS metabolism in root and shoot tissues

The ROS and RNS-inducing effects of Se were compared in the organs of *Astragalus* species (Fig 5) in order to reveal the possible link between Se tolerance or sensitivity and Se-induced oxidative and nitrosative (together nitro-oxidative) stress.

In root tips of *A. membranaceus*, both Se concentrations increased superoxide levels, although the highest and significant superoxide production was observed in case of 50 μM Se resulting in 170% increase (Fig 5AB). In the root tips of tolerant *A. bisulcatus*, selenate had no effect on superoxide levels (Fig 5 AB). In intact cotyledons, superoxide levels were examined qualitatively by NBT staining (Fig 5C). In case of 50 or 100 μM Se-treated *A. membranaceus* plants, the intense presence of blue colorization indicated superoxide production. In *A. bisulcatus*, slightly intensified blue staining was detected only as the effect of 100 μM Se treatment (Fig 5C). In order to reveal the mechanism of the different superoxide-response of the species, we examined the metabolism of this reactive intermediate. The superoxide-generating NADPH oxidase (NOX) isoenzymes were separated by native-PAGE and a protein band being strongly present in all samples was determined (Fig 5D "main band"). In the cotyledons of *A. bisulcatus* one, while in *A. membranaceus* four additional putative NOX isoenzymes were detected. As the effect of Se, only slight changes occurred in NOX isoenzyme activities especially in *A. bisulcatus*, while more protein bands showed increased activity in *A. membranaceus* cotyledons (Fig 5D, Suppl Fig 3). In the roots of both species, the activity of the main NOX protein band was less pronounced; although Se induced its activity in *A. bisulcatus* roots. In addition to the main protein band, four other isoenzymes were detected in *A. bisulcatus* roots, three of which showed induction as the effect of selenate exposure (Fig 5D, Suppl Fig 3). In case of *A. membranaceus* roots, Se reduced the activity of the main NOX band, which seemed to be substituted by the appearance and strong activation of additional, putative NOX isoenzymes (indicated by asterisks in Fig 5D, Suppl Fig 3).

Both concentrations of selenate caused notable (~30% and 38%) induction of superoxide-eliminating SOD enzymes in *A. membranaceus* roots, while the effect of selenate in the root system of *A. bisulcatus* proved to be much slighter (~10%, Fig 5E). Regarding the cotyledons,

selenate exposure resulted in SOD activation only in *A. membranaceus* and the effect proved to be slighter compared to the root (~15%, Fig 5F).

We separated SOD isoforms by native-PAGE, and differences were observed between the species and also between the organs (Fig 5E). In both organs of *A. bisulcatus*, four activity bands (MnSODI, FeSOD I, FeSOD II, Cu/Zn SOD) were identified, while in *A. membranaceus* cotyledons six bands were detected (MnSODII, FeSOD I, FeSOD II, Cu/Zn SOD I, Cu/Zn SOD II, Cu/Zn SOD III). Moreover, in the root system of *A. membranaceus* only three SOD activity bands (MnSODII, FeSODI, FeSODII) were observed. Quantification showed, that selenate at the largest applied concentration exerted slight effect on SOD isoenzymes in *A. bisulcatus* cotyledons (Suppl Fig 4). In contrast, five SOD isoforms of six showed intensified activity as the effect of 50 μM Se in cotyledons of *A. membranaceus*. Regarding to the root system, both applied Se treatments induced the activity of Mn, Fe, Cu/Zn SODs in *A. bisulcatus*, but these inductions were much intense in *A. membranaceus* (Suppl Fig 4).

Similar to superoxide, nitric oxide formation significantly enhanced as the effect of 50 μM Se in the root of sensitive *A. membranaceus* (Fig 6 AB). Regarding peroxynitrite, 50 μM Se resulted in its accumulation, but the highest Se dose decreased its level in the root tips of the sensitive species (Fig 6 EF). Interestingly, none of the applied selenium treatments had any observable effect on the examined RNS levels in *A. bisulcatus* root tips (Fig 6 AB and EF).

Unlike the roots, both species showed NO accumulation in their cotyledons as the effect of 50 μM Se (Fig 6 CD). Both Se concentrations triggered significant peroxynitrite generation in the cotyledons of *A. membranaceus*, while in the tolerant species only slight, non-significant changes were observed (Fig 6 GH). Selenium-induced alterations in GSNO levels were also determined in the root and shoot tissues of the species (Fig 6 I-L). Under control conditions, significantly higher GSNO content was determined in both organs of *A. bisulcatus* compared to *A. membranaceus*. Selenate treatments caused significant reduction in GSNO levels of both

A. bisulcatus organs. Similar Se-induced diminution of GSNO content was found in *A. membranaceus* roots (Fig 6 IJ); however, in the cotyledons Se exposure led to the significant and concentration-dependent increase of GSNO levels (Fig 6 KL). Significantly increased fluorescence was detected in GSNO pre-treated sections, which served as positive controls, while light-inactivated GSNO did not result in fluorescence increase (Suppl Fig 7). In their cotyledons, both species showed relatively high S-nitrosoglutathione reductase (GSNOR) activity compared to the root system during control conditions (Fig 6 M, Suppl Fig 6). Selenate exerted inhibitory effect on GSNOR activity in *A. bisulcatus* cotyledons, while notably induced it in the cotyledons of 50 μM selenate-treated *A. membranaceus*. As for the control root system, *A. bisulcatus* showed higher GSNOR activity than *A. membranaceus* where the activity was barely detectable (Fig 6M, Suppl Fig 6). In case of *A. bisulcatus*, selenate exerted concentration-dependent reducing effect on GSNOR activity. As opposed to this, selenate did not modify the enzyme activity in the root of *A. membranaceus* (Fig 6M, Suppl Fig 6).

Selenium-induced protein tyrosine nitration

Protein tyrosine nitration as a consequence of RNS accumulation was investigated by both immunofluorescence (Fig 7) and western blot analysis (Fig 8). In cross sections of *A. membranaceus* primary roots, immunofluorescent signal related to 3-nitrotyrosine was observable mainly in endodermal cell layer and within the central cylinder (Fig 7B). Selenium exposure led to the significant increase in 3-nitrotyrosine-dependent fluorescent signal in all tissues of the root (Fig 7A), but this elevation was the most pronounced in the central cylinder (Fig 7B). Under control conditions, 3-nitrotyrosine located mainly in the endodermal cell layer of *A. bisulcatus* roots (Fig 7B). Milder Se treatment caused a slight increase of the fluorescence in the endodermis and the most serious Se exposure induced 3-nitrotyrosine accumulation in all tissues of the primary root, although this increase was smaller than in *A. membranaceus*

roots (Fig 7A). In cotyledons, *Astragalus* species showed differences in physiological 3-nitrotyrosine levels, since *A. bisulcatus* showed higher 3-nitrotyrosine-related fluorescence (Fig 7C). Moreover, high levels of 3-nitrotyrosine were found to be located in cotyledon veins (Fig 7D). Both selenate treatments significantly decreased the 3-nitrotyrosine content of *A. bisulcatus* cotyledons but in case of A. *membranaceus*, 100 μM selenate induced 3-nitrotyrosine formation (Fig 7C). As positive and negative controls, sections were treated with SIN-1 and enhanced fluorescence intensity was detected while urate pre-treatment remarkably mitigated 3-nitrotyrosine-dependent fluorescence (Suppl Fig 7).

In whole protein extract, tyrosine nitration was determined by western blot analysis (Fig 8). In *A. membranaceus* cotyledons, selenate intensified tyrosine nitration of five protein bands (~27, 22, 17, 12, 10 KDa, indicated by grey arrows) but newly nitrated protein band could not be observed. In cotyledons of *A. bisulcatus*, both Se treatments resulted in the appearance of a highly nitrated protein band (with high molecular weight, indicated by black arrows) but Se did not cause any other nitration-related change in the proteome. In *A. bisulcatus* roots, Se did not intensify protein tyrosine nitration, even caused decrease in three protein bands (~75, 12, 10 KDa). In contrast, the Se-sensitive *Astragalus* species showed several protein bands which immunopositivity towards anti-3-nitrotyrosine showed Se-dependent appearance.

DISCUSSION

Both species were able to take up selenate from the external media (Fig 1). Even though *A. membranaceus* accumulated large amount of Se in its root, the root-to-shoot Se translocation proved to be slight. In contrast, in *A. bisulcatus* cotyledons, more than 7-fold Se concentrations were measured compared to *A. membranaceus* indicating a high rate of Se translocation. Indeed, the root-to-shoot Se ratio was 3.8 in *A. bisulcatus* plants grown on 100 μM selenate suggesting that it is a hyperacumulator species (Freeman *et al.,* 2010). Furthermore, the relative high endogenous Se content in the organs of control *A. bisulcatus* indicates its hyperaccumulator nature. Also the amount of the accumulated Se (~1800 μg g^{-1} DW in the cotyledons of 100 μM selenate-exposed plants) supports hyperaccumulation capability of *A. bisulcatus* (Mehdawi and Pilon-Smits, 2012). In addition to Se, exogenous selenate affected the concentrations of essential microelements like Fe, Zn, Mn and B (Table 1) especially in *A. membranaceus* inhibiting their absorption and consequently causing disturbances in their homeostasis. Similar antagonism between Se and macro- or microelements has earlier been described by others (Pazurkiewicz-Kocot *et al.,* 2003; Zembala *et al.,* 2010; Filek *et al.,* 2010). Reduced availability of essential microelements may worsen growth and physical condition of the plant. Boron is needed to maintain cell wall integrity, while Zn protects membrane lipids and proteins and together with Mn, Cu and Fe is the metal component of SOD antioxidant enzymes (Cakmak, 2000). In case of the Se hyperaccumulator *A. bisulcatus* the microelement homeostasis seems to be more stable, since Se did not cause disturbance in it which may contribute to the better tolerance of this species.

Selenium negatively affected the germination capability and the biomass production of young *A. membranaceus,* but the germination and growth of *A. bisulcatus* proved to be insensitive to selenium (Fig 2). Although, root elongation concentration-dependently decreased as the effect of elevating Se concentrations suggesting the higher sensitivity of the root system

to Se compared to the aerial plant parts (Lehotai *et al.,* 2016). Because of the Se concentration-dependent response of elongation, root growth can be used as an indicator of selenium tolerance (Tamaoki *et al.,* 2008; Molnár *et al.,* 2018a). The hyperaccumulator *A. bisulcatus* was able to maintain its root growth on Se-containing medium (Fig 3A) even though meristem cells suffered certain degree viability loss (Fig 3BC). The reduced root elongation (Fig 3A) and meristem viability (Fig 3BC) of *A. membranaceus* indicates its sensitivity to Se. Beyond the viability of the root apical meristem, in the background of Se-inhibited organ development, the disturbances of hormone homeostasis or unfavourable alterations in primary metabolism can also be determined (reviewed Kolbert *et al.,* 2016). Based on the observed parameters (germination, biomass production, root elongation, cell viability), young *A. membranaceus* proved to be Se sensitive, while the hyperaccumulator *A. bisulcatus* showed remarkable Se tolerance which supports the previously described connection between Se hyperaccumulation and (hyper)tolerance (Mehdawi and Pilon-Smits, 2012). The main reason for Se tolerance of *A. bisulcatus* is that this species expresses SMT enzyme which prevent toxic seleno-amino acid formation (Neuhierl and Bock, 1996). Considering the high shoot Se accumulation (Fig 1B), it can be assumed that the notable Se tolerance of *A. bisulcatus* is due to detoxification and not exclusion.

We observed selenium-induced alterations in root structure of both *Astragalus* species. Thicker roots of control and 50 μM Se-treated sensitive *A. membranaceus* compared to *A. bisulcatus* were probably due to the thicker cortex (Fig 4AB). The increment of the root diameter, including the thickening of the cortex is common in heavy metal stressed plant roots (Arduini *et al.,* 1995; Maksimović *et al.,* 2007; Potters *et al.,* 2007). The hyperaccumulator species, *A. bisulcatus* showed more intense Se-induced root thickening than *A. membranaceus* (Fig 4 ABC), which is in agreement with the results of Li *et al.* (2009) where in the hyperaccumulating ecotype of *Sedum alfredii,* lead/zinc-triggered increment in root diameter

and other root morphological parameters was observed. The deposition of callose seems to be a good marker of stress induced cell wall alterations. It was formerly found that copper can induce callose formation in onion epidermal cells and in the root tips of *Brassica* species (Kartusch 2003; Feigl *et al.*, 2013). In our study, the sensitive *Astragalus* species showed both Se-triggered callose accumulation (Fig 4D) and exodermal suberin lamellae deposition (Fig 4E, Dalla Vecchia *et al.*, 1999; Rahoui *et al.*, 2017) which together may serve as an extracellular barrier limiting water and mineral uptake. This may result in Se exclusion and at the same time the inhibition of growth. In case of *A. bisulcatus*, not only the exodermis but also the endodermis exhibited the presence of suberin (Fig 4E). Since exodermal suberin deposition occurs earlier in time followed by the appearance of endodermal suberin as the effect of metal stress (Vaculík *et al.*, 2012), we can conclude that in case of *A. membranaceus*, the delayed formation of Se-induced endodermal suberin lamellae formation is associated with Se sensitivity. Moreover, the development of apoplastic barriers (exodermal and endodermal) can be considered as an adaptive trait (Vaculík *et al.*, 2012).

For the toxic effect of Se the accumulation of ROS and the consequent oxidative stress is partly responsible (Van Hoewyk, 2013). The accumulation of the rapidly generating, harmful ROS, superoxide anion (Fig 5 ABC) as well as the induction of SOD activity (Fig 5 EF) suggest Se-triggered oxidative stress in *A. membranaceus* organs while no sign of serious oxidative damage was observed in *A. bisulcatus*. The expression of superoxide generating NOX isoenzymes showed species-specificity in *A. membranaceus* roots, and newly expressed NOX isoenzymes were observed as the effect of selenate (Fig 5D). Regarding SOD isoenzymes, *A. membranaceus* cotyledons express more Cu/Zn SODs than *A. bisulcatus* and selenate remarkably increased the activity of most of the isoenzymes (Fig 5G). Selenium-triggered superoxide accumulation has been observed in the non-accumulator *Stanleya albescens* and *Arabidopsis thaliana* and in secondary accumulators like *Brassica napus*, *Brassica rapa* and

Brassica juncea (Freeman *et al.*, 2010, Tamaoki *et al.*, 2008, Dimkovikj and Van Hoewyk, 2014; Chen *et al.*, 2014; Molnár *et al.*, 2018ab). In the hyperaccumulator species *Stanleya pinnata*, elevated levels of ROS scavenging compounds (ascorbate and glutathione) were observed which are involved in the prevention of selenium-induced oxidative stress (Freeman *et al.*, 2010). In our study, *A. bisulcatus* showed moderately higher SOD activities (especially Cu/Zn SODs) in the roots compared to *A. membranaceus* (Fig 5F) which may contribute to endurance against Se-induced oxidative stress. At the same time, Se hyperaccumulators are known to accumulate organic selenium forms (mainly methyl-seleno-cysteine) instead of the oxidative stress-inducing inorganic Se compounds which may be a relevant protection mechanism against oxidative stress (Schiavon and Pilon-Smits, 2017).

Additionally, Se exposure has been earlier shown to disturb the metabolism of RNS. Milder selenate dose triggered NO production mainly in the non-accumulator species (Fig 6 A-D) similarly to selenite-exposed *Pisum sativum* (Lehotai *et al.*, 2016) or selenate-treated secondary accumulator *Brassica rapa* (Chen *et al.*, 2014). Based on the results of Rios *et al.* (2010) it is conceivable that selenate induces nitrate reductase (NR) which is the main enzymatic NO source in the root system and is also involved in NO production in the aerial plant parts (Zhang *et al.*, 2011). The effect of selenium on NR activity can be direct or indirect since Se-induced S-deficiency may increase Mo content thus inducing NR (Shinmachi *et al.*, 2010, Yu *et al.*, 2010). In our experiments, significantly higher Mo concentrations were measured in both organs of selenate-treated A. *membranaceus* (Table 1) which can be connected to the elevated NO production. Peroxynitrite can be formed *in vivo* in the fast reaction between superoxide radical and NO (Kissner *et al.*, 1997) thus their accumulation may predict and explain Se-induced $ONOO^-$ generation. The concentration of this strong oxidative and nitrosative agent could reflect overall stress severity (Arasimowicz-Jelonek and Floryszak-Wieczorek, 2011) therefore we can suspect that *A. membranaceus* suffers more severe Se-

triggered nitro-oxidative stress compared to *A. bisulcatus*. However, as the effect of the highest Se dose in *A. membranaceus* root, peroxynitrite level decreases (Fig 6 E) due to the possible activation of scavenging mechanisms. GSNO is a mobile NO storage in plants being responsible for protein S-nitrosylation. The spontaneous decomposition of GSNO leads to NO production while it is enzymatically reduced by GSNOR or it can catalyse the transnitrosylation of protein thiols leading to its decomposition (Lindermayr, 2018; Begara-Morales *et al.*, 2018). Both species responded to the presence of selenate by decreasing the endogenous GSNO reservoir of their roots (Fig 6 I), however this resulted in NO accumulation only in *A. membranaceus* (Fig 6 A). Presumably, in *A. bisulcatus* the originally high GSNO content participated in transnitrosylation reactions with cysteine thiols in proteins leading to S-nitrosothiol (SNO) formation and GSNOR-catalysed reduction is not involved in GSNO metabolism under Se stress. In the cotyledon of *A. bisulcatus*, the level of GSNO decreased (Fig 6 KL) possibly due to spontaneous decomposition yielding NO but not to GSNOR activity.

Similar to other species (reviewed by Corpas *et al.*, 2013a), both *A. membranaceus* and *A. bisulcatus* can be characterized by a certain physiological nitropoteome which means that a part of their protein pool is nitrated even at control state. Both the Se-induced increase in fluorescence intensity (Fig 7) and the presence of several newly nitrated protein bands (Fig 8) indicated more intense protein tyrosine nitration in the organs of *A. membranaceus* compared to the hyperaccumulator *A. bisulcatus*. Moreover, both immunofluorescence and western blot results showed that the tolerant species possesses large physiological nitroproteome as well as large mobile NO storage (GSNO) with which it is able to buffer NO radical content. The selenium-induced GSNO and 3-nitro-tyrosine decompositions without the accumulation of the reactive $^{\cdot}$NO may contribute to tolerance against nitro-oxidative stress in *A. bisulcatus*. The Se-triggered decrease in the amount of 3-nitrotyrosine may be conceivable *via* proteasomal degradation (Castillo *et al.*, 2015).

Our experiments examined the sensitivity of young non-accumulator *A. membranaceus* and hyperaccumulator *A. bisulcatus* to selenium in connection to secondary oxidative and nitrosative processes and the obtained results are summarized in Fig 9. As expected, the observed parameters (Se accumulation, microelement homeostasis, tissue-level changes in the roots, germination, biomass production, root growth, cell viability) indicated that *A. membranaceus* is Se sensitive while *A. bisulcatus* tolerates the presence of high selenium doses. We first revealed that in *A. membranaceus,* Se sensitivity coincides with the Se-induced disturbance of superoxide metabolism involving NOXs and SODs leading to superoxide accumulation. Furthermore, this study points out for the first time that Se induced the production or disturbed the metabolism of RNS (NO, ONOO-, GSNO) consequently resulting in intensified protein tyrosine nitration in the sensitive *A. membranaceus*. In the (hyper)tolerant and hyperaccumulator *A. bisulcatus*, Se decreased large GSNO content and tyrosine nitroproteome without the accumulation of NO radical resulting in the lack of tyrosine nitration. These suggest that this species is able to prevent Se-induced nitro-oxidative stress to which enhanced ROS/RNS scavenging capability may also contribute. Given that the elevated levels of other elements (e.g. zinc, arsenic, cadmium) have been reported to induce protein nitration and cause similar disturbances in ROS and RNS metabolism like selenium (Feigl *et al.*, 2015; Feigl *et al.*, 2016; Letterier *et al.*, 2012; Liu *et al.*, 2018), excess selenium-induced nitro-oxidative stress can be considered rather a general than a Se-specific phenomenon. Future research should focus on the evaluation of the antioxidative system in order to get more accurate view about nitro-oxidative processes in relation to Se tolerance.

MATERIALS & METHODS

Plant material and growing conditions

Astragalus bisulcatus (Hook.) A. Gray seeds were obtained from B&T World Seeds (Aigues-Vives, France) and *Astragalus membranaceus* (Fisch.) Bunge seeds were provided by Professor Aaron Chang (Kaohsiung Medical University, Graduate Institute of Natural Products, Kaohsiung, Taiwan).

Seeds were surface sterilized with 20 % (v/v) sodium hypochlorite for 20 minutes, and were washed with sterile distilled water for four times in 20 minutes. Seeds were dried on a sterile metal filter and we polished them one by one using P-400 sanding paper in order to scratch the external seed coat. Seeds were placed on agar medium (the scratched surface of the seeds contacted the medium). Plastic, square Petri dishes contained half-strength Murashige-Skoog medium (0.8% v/v agar, 1% sucrose) supplemented with 0 (control), 50 or 100 μM sodium selenate (Na_2SeO_4). Both plant species were grown during controlled conditions (150 μmol m^{-2} s^{-1} photon flux density, 12h/12h light/dark cycle, relative humidity 55–60% and temperature 25±2 ºC) for 14 days. All chemicals were purchased from Sigma-Aldrich (St. Louis, USA) unless stated otherwise.

Se and microelement content analysis

Cotyledon and root materials of both *Astragalus* species were harvested separately and rinsed with distilled water then dried at 70 ºC for 72 hours. Nitric acid (65% w/v, Reanal, Budapest, Hungary) and hydrogen peroxide (30%, w/v, VWR Chemicals, Poole, England) were added to dried plant material. The samples were destructed in microwave destructor (MarsX-press CEM, Matthews, USA) at 200 ºC and 1600 W for 15 min. After appropriate dilutions with distilled water, the samples were transferred to 20 mL Packard glasses. Element concentrations were determined by inductively coupled plasma mass spectrometry (ICP-MS) (Agilent 7700

Series, Santa Clara, USA). Concentrations of Se and essential microelements (Fe, Zn, Mn, Mo, B) are given in μg g^{-1} dry weight (DW). These analyses were carried out two times with three samples each (n=3).

Evaluation of germination, growth parameters, root cell viability and Se tolerance index

Germinated seeds were counted in each Petri dish and germination percentages (%) were calculated. Fresh weights of root and shoot materials were measured using a balance and the values are given in mg. Length of primary roots were measured manually. From the data selenium tolerance index (%) was calculated according to the following formula: tolerance index (%)= (treated root length/mean control root length) * 100

Cell viability in root apical meristem was determined by using fluorescein diacetate (FDA) fluorophore. Root tips were incubated in 10 μM FDA solution (prepared in 10/50 mM MES/KCl buffer, pH 6.15) for 30 min in darkness and were washed four times in buffer.

These data were acquired from three separate generations and in each generation 15 plants/seeds were examined (n=15).

Evaluation of tissue-level changes in the roots induced by selenium

Small pieces of root samples derived from the mature zone were fixed in 4 % (w/v) paraformaldehyde according to Barroso et al. (2006). After the fixation root samples were washed in distilled water and embedded in 5% agar (bacterial; Zelko *et al.*, 2012 with modifications). Then 100 μm thick cross sections were prepared using a vibratome (VT 1000S, Leica, Wetzlar, Germany). The sections were placed on a slide with a drop of water and were stained with aniline blue (AB; 0.5 % w/v) to detect the deposition of callose. The root sections were observed by light microscope and inverted fluorescent microscope (Zeiss Axiovert 200

M, Carl Zeiss, Jena, Germany) equipped with a digital camera (AxiocamHR, HQ CCD, Carl Zeiss, Jena, Germany). Images obtained by light microscopy were applied to measure several parameters of the root such as root diameter, the thickness of the cortex and the diameter of the stele according to Arduini et al. (1995). All data are given in µm.

Fluorescent microscopy was applied to observe the fluorescence of secondary cell wall compounds like lignin and suberin (Auramine O staining) as well as the formation of callose as a result of Se stress, using filter set 9 (exc.:450–490 nm, em.:515–∞ nm) and filter set 49 (exc.: 365 nm, em.: 445/50 nm) (Feigl *et al.*, 2013; Rahoui *et al.*, 2017). In both cases, fluorescence intensity (pixel intensity) was measured on digital images applying Axiovision Rel. 4.8 software (Carl Zeiss, Jena, Germany) within circles of 100 µm radii which were set to cover the largest area of the vascular cylinder. The data of the Se-treated plants were calculated in control %.

These experiments were carried out on two separate plant generations with 6 plants examined each (n=6).

In situ detection of ROS and RNS in the root tips and in cotyledons

Dihydroethidium (DHE) at 10 µM concentration was applied for the detection of superoxide anion levels in the roots. Root segments were incubated for 30 min in darkness at 37 ºC, and washed two times with Tris-HCl buffer (10 mM, pH 7.4) (Kolbert *et al.*, 2012). In cotyledons, instead of DHE, nitroblue-tetrazolium (NBT) was used for visualizing superoxide production. Excised cotyledons were incubated in Falcon tubes containing 5 mL NBT solution (1 mg mL^{-1} in 10 mM phosphate buffer, pH 7.4) for 30 min under illumination. Pigments were removed by incubating the cotyledons in 80% (v/v) ethanol at 70 °C for 30 minutes.

Nitric oxide level of the root tips and in handmade cross-sections from cotyledons was monitored with the help of 4-amino-5-methylamino- 2′,7′-difluorofluorescein diacetate (DAF-

FM DA) according to Kolbert et al. (2012). Root and cotyledon segments were incubated in 10 µM dye solution for 30 min (darkness, 25±2 °C), and washed twice with Tris-HCl (10 mM, pH 7.4).

Peroxynitrite was visualised also in root tips and in handmade cross sections of cotyledons. Samples were incubated in 10 µM dihydrorhodamine 123 (DHR) prepared in Tris-HCl buffer. After 30 min of incubation at room temperature, root tips and cotyledon segments were washed two times with the buffer solution (Sarkar *et al.*, 2014).

These analyses were carried out two times with 10 samples each (n=10).

Determining SOD, NADPH oxidase izoenzymes and GSNOR activity by native PAGE

Fresh cotyledon and root tissues of *A. bisulcatus* and *A. membranaceus* were grounded with double volume of extraction buffer (50 mM Tris–HCl buffer pH 7.6–7.8) containing 0.1 mM EDTA, 0.1% Triton X-100 and 10% glycerol and centrifuged at 12,000 rpm for 20 min at 4 °C. The protein extract was treated with 1% protease inhibitor cocktail and stored at -20 °C. Protein concentration was determined using the Bradford (1976) assay with bovine serum albumin as a standard. In order to avoid the effect of the changes in protein concentration and composition induced by the treatments, our data are standardized to fresh weight by loading equal volumes of protein extracts in each well. Silver staining was performed according to Blum et al. (1987) with slight modifications. The gel was fixated with methanol and acetic acid, then treated with a sensitizing solution and staining solution containing $AgNO_3$. The gel was developed in a solution containing sodium carbonate and formaldehyde (Suppl. Fig 2 and Suppl Fig 5).

NADPH oxidase (NOX) activity was examined on 10% native polyacrylamide gels by the NBT reduction method of López-Huertas et al. (1999) with slight modifications. In case of

cotyledons 15 µl and in case of roots 25 µl protein extracts were loaded in each well. Following electrophoresis, the gel was incubated in reaction buffer (50 mM Tris-HCl pH 7.4, 0.1 mM $MgCl_2$, 1mM $CaCl_2$) containing 0.2 mM NBT and 0.2 mM NADPH for 20 minutes in darkness. As positive control, NADPH oxidase specific inhibitor diphenylene iodonium (DPI) was used at a final concentration of 50 µM. In addition, NADPH-independent superoxide production was examined on a gel without NAPDH supplementation.

SOD activity was measured based on the ability of the enzyme to inhibit photochemical reduction of nitro blue tetrazolium (NBT) catalysed by riboflavin, as described by Dhindsa et al. (1981). 250 mg of plant biomass was grounded with 10 mg polyvinyl polypyrrolidone (PVPP) in 1 ml 50 mM pH 7.0 phosphate buffer containing 1 mM of EDTA. The enzyme activity is expressed in specific activity (U/g fresh weight), where on unit of SOD activity means 50% inhibition of NBT reduction in light.

For the examination of SOD activity and isoenzymes, protein extracts (15 µl and 25 µl in case of cotyledons and roots, respectively) were subjected to native gel electrophoresis on 10 % polyacrylamide gel (Beauchamp and Fridovich, 1971). The gel was rinsed in 50 mM potassium phosphate buffer (pH 7.8) two times, then incubated for 20 minutes in 2.45 mM NBT in darkness then for 15 minutes in freshly prepared 28 mM TEMED solution containing 2.92 µM riboflavin. After the incubation, the gels were washed two times and developed by light exposure. SOD isoforms were identified by incubating gels in 50 mM potassium phosphate containing 2 mM potassium cyanide to inhibit Cu/Zn SOD activity or 5 mM H_2O_2 which inhibits Cu/Zn and Fe SOD activity for 30 min before staining with NBT. Mn SODs are resistant to both inhibitors.

GSNOR activity was visualised using a slightly modified method described by Seymour and Lazarus (1989). Native polyacrylamide gel electrophoresis was performed using 6% acrylamide gels with Tris-boric-EDTA buffer (8.9 mM Tris base, 8.9 mM boric acid and 0.2

mM Na_2EDTA, pH 8). In case of cotyledons 30 µl and in case of roots 50 µl protein extracts were loaded in each well. Gels were incubated for 15 minutes at 4 °C in the presence of 2 mM NADH solution prepared in 100 mM sodium phosphate buffer (pH 7.4). Excess buffer was removed and a filter paper containing freshly prepared 3 mM GSNO solution (prepared in 100 mM sodium phosphate buffer, pH 7.4) was added (15 min, darkness, 4 °C). NADH UV fluorescence was visualised at 312 nm wavelength using a gel documentation system (Image System Felix 1000/2000, Biostep, Burkhardtsdorf, Germany). GSNOR enzyme activity consumed NADH resulting in dark bands in the gel.

Relevant bands showing NOX, SOD or GSNOR signals were quantified by Gelquant software (provided by biochemlabsolutions.com) and the data are presented as Suppl Fig 3,4 and 6, respectively.

These experiments were carried out on two separate plant generations with 3 samples examined each (n=3).

Immunofluorescent detection of GSNO and 3-nitro-tyrosine in root and cotyledon cross sections

Cross sections were prepared using a vibratome as described earlier and immunodetection was performed according to Corpas et al. (2008) with slight modifications. Free-floating sections were incubated at room temperature overnight with rat antibody against GSNO (VWR Chemicals, Poole, England) diluted 1:2500 in TBSA-BSAT solution containing 5 mM Tris buffer (pH 7.2), 0.9% (w/v) NaCl, 0.05% (w/v) sodium azide, 0.1% (w/v) bovine serum albumin (BSA) and 0.1% (v/v) Triton X-100. Samples were washed three times with TBSA-BSAT solution within 15 min. After the washings, cross sections were incubated with FITC-conjugated rabbit anti-rat IgG secondary antibody (1:1000 in TBSA-BSAT, Agrisera,

Vännäs, Sweden) for one hour at room temperature. Samples were placed on microscopic slides in PBS:glycerine (1:1). As a positive control, cross-sections were treated with 250 μM GSNO (prepared in TBSA-BSAT) for one hour prior to the labelling process. Light-inactivated GSNO was prepared as described by Wodala and Horváth (2008) and was applied for one hour prior to labelling.

Immunodetection of 3-nitro-tyrosine was carried out according to Valderrama et al. (2007). Samples were incubated for 3 days at 4 °C with polyclonal rabbit antibody against 3-nitrotyrosine (Sigma-Aldrich, St. Louis, USA) diluted in TBSA-BSAT (1:300). After three washings with TBSA-BSAT, sections were incubated for 1h at room temperature in FITC-conjugated goat anti-rabbit IgG (1:1000 in TBSA-BSAT, Agrisera, Vännäs, Sweden). Samples were placed on microscopic slides in PBS:glycerine (1:1). As a positive control, samples were incubated with 3-morpholino-sydnonimine (SIN-1, 1 mM in TBSA-BSAT) for one hour prior to the labelling process. Urate at 2 mM concentration (prepared in distilled water) was applied for one hour prior to the labelling process in order to quench endogenous peroxynitrite.

All microscopic analysis was accomplished under Zeiss Axiovert 200 M inverted microscope (Carl Zeiss, Jena, Germany) equipped with a digital camera (AxiocamHR, HQ CCD, Carl Zeiss, Jena, Germany). Filter set 10 (exc.: 450–490, em.: 515–565 nm) was used for FDA, DAF-FM, DHR and FITC, filter set 9 (exc.:450–490 nm, em.:515–∞ nm) for DHE and filter set 49 (exc.: 365 nm, em.: 445/50 nm) was applied for UV autofluorescence. Pixel intensity was measured in area of circles using Axiovision Rel. 4.8 software (Carl Zeiss, Jena, Germany). The radii of circles were set to cover the largest sample area.

Immunofluorescent detections were carried out on two separate plant generations with 5-6 plants examined each (n=5-6).

Detection of nitrated proteins using SDS-PAGE and western blot

Protein extracts were prepared as described earlier. To evaluate the electrophoresis and transfer we used Coomassie Brilliant Blue R-350 according to Welinder and Ekblad (2011). As a protein standard, actin from bovine liver (Sigma-Aldrich, cat. no. A3653) was used (Suppl Fig 8). Silver staining was carried out as previously described.

25 μg of denaturated root and shoot protein were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12 % acrylamide gels. The proteins were transferred to PVDF membranes using the wet blotting procedure (25 mA, 16h) for immunoblotting. After transfer, membranes were used for cross-reactivity assays with rabbit polyclonal antibody against 3-nitrotyrosine diluted 1:2000. Immunodetection was performed by using affinity isolated goat anti-rabbit IgG-alkaline phosphatase secondary antibody in dilution of 1:10000, and bands were visualized by using NBT/BCIP reaction. Nitrated bovine serum albumin served as positive control. Western blot was applied to 2 separate protein extracts from different plant generations, multiple times per extract, meaning a total of 6 blotted membranes (n=2).

Statistical analysis

Root morphological data (Fig 4) were analysed using STATISTICA 10.0 software. To ascertain the effect of Se treatment on the anatomical parameters examined one-way analysis of variance (ANOVA) was applied. Since most of the data showed non-normal distribution, we took a non-parametric test (Kruskal-Wallis ANOVA) to test the differences of means. In order to determine the relationship between Se concentration and the measured parameters, a non-parametric analysis of correlation (Spearman's Rank Order Correlation) was used. Data are given as mean values ± standard deviation (SD), the level of significance was * $p<0.05$, ** $p<0.01$ and *** $p<0.001$. In case of any additional data the results are shown as mean±SE. Data

were statistically evaluated by Duncan's multiple range test (One-way ANOVA, $P \leq 0.05$) using SigmaPlot 12 or by Student's T-test applying Microsoft Excel 2010.

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SUPPLEMENTARY INFORMATION

Suppl Fig 1 Cell viability (control%) in root tips of *Astragalus* species treated with 0, 50, 75 or 100 μM sodium selenate for 3, 7, 11 and 14 days (n=10). On the 3rd and 7th days, the viability of both species remarkably decreased and the difference between the viability of the species proved to be small as well as the difference caused by Se treatment concentrations. For the 11th day, the viability of the tolerant species increased and a good correlation between concentrations and viability could be seen. Meanwhile the sensitive plant showed further viability loss for the 14th day, and the tolerant and the sensitive species were well separated in terms of their viability as well as the clear Se concentration-dependence of root cell viability could be observed.

Suppl Fig 2 Silver-stained native gel (10%) as a control for NADPH oxidase activity gel. The gel shows a good run-off, and major protein bands do not show any greater decomposition in the protein extract. It has to be noted; however, that in 100 μM Se-treated *A. membranaceus* root, the formation of selenoproteins may trigger protein turnover.

Suppl Fig 3 Quantification of NADPH oxidase in gel activities using Gelquant software. Because of the several isoforms the position of "the main band" was determined as "0". The izoforms which are slower compared to the main band are labelled with positive numbers, while the faster isoforms are indicated with negative numbers in order to indicate their position within the gel. The obtained intensities are depicted in graphs.

Suppl Fig 4 Quantification of SOD in gel activities using Gelquant software. The values of the individual isoforms are depicted on separate graphs except Cu/Zn SOD isoforms in *A. membranaceus* cotyledon.

Suppl Fig 5 Silver-stained native gel (6%) as a control for GSNOR activity gel. The gel shows a good run-off, and major protein bands do not show any greater decomposition in the protein

extract. It has to be noted; however, that in 100 μM Se-treated *A. membranaceus* root, the formation of selenoproteins may trigger protein turnover.

Suppl Fig 6 Quantification of GSNOR in gel activities using Gelquant software. The data are depicted in graphs.

Suppl Fig 7 Representative images showing root cross sections (A,B,C,G,H,I) and cotyledon cross sections (D,E,F,J,K,L) labelled for GSNO (A-F) or 3-nitrotyrosine (G-L) immunodetection. Cross sections were prepared from the organs of 14-days-old *A. bisulcatus* grown on half-strength MS medium under control conditions. Immunolocatization of GSNO in control root cross-section (A), in control cotyledon cross section (D) and in root and cotyledon cross sections pre-treated with 250 μM GSNO (B and E) or 250 μM decomposed GSNO (C and F) for one hour prior labelling. 3-nitro-tyrosine immunodetection in control root cross-section (G), in control cotyledon cross section (J) and in root and cotyledon cross sections pre-treated with 1 mM SIN-1 (H and K) or 2 mM urate (I and L) for one hour prior labelling. Mean values of pixel intensities and standard errors are indicated. Bars=200 or 500 μm.

Suppl Fig 8 *Astragalus* proteins separated by SDS gel electrophoresis and transferred to PVDF membrane. Actin from bovine was used as standard. In both organs of both species, actin bands are observable, which proves the intactness of the samples.

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Table 1 Concentrations of Fe, Zn, Mn, B and Mo in the root system and cotyledons of 14-days-old *Astragalus* species treated with 0, 50 or 100 µM selenate for 14 days. Different letters indicate significant differences according to Duncan-test (n=3, P≤0.05).

		Fe (µg g^{-1} DW)			Zn (µg g^{-1} DW)			Mn (µg g^{-1} DW)			B (µg g^{-1} DW)			Mo (µg g^{-1} DW)		
		Control	**50 µM Se**	**100 µM Se**	**Control**	**50 µM Se**	**100 µM Se**	**Control**	**50 µM Se**	**100 µM Se**	**Control**	**50 µM Se**	**100 µM Se**	**Control**	**50 µM Se**	**100 µM Se**
A. membranaceus	**cotyledon**	133.20 ± 1.9^{a}	65.35 ± 0.6^{b}	59.81 ± 1.6^{c}	101.50 ± 1.2^{a}	72.18 ± 1.4^{b}	65.54 ± 0.3^{c}	106.61 ± 0.4^{a}	85.94 ± 0.7^{b}	68.22 ± 1.1^{c}	113.84 ± 0.7^{a}	53.22 ± 0.5^{b}	39.61 ± 1.0^{c}	5.87 ± 0.08^{b}	6.80 ± 0.1^{a}	6.88 ± 0.09^{a}
	root	503.80 ± 8.3^{a}	338.81 ± 11.3^{b}	358.90 ± 16.3^{b}	152.10 ± 1.6^{b}	145.51 ± 2.1^{c}	166.93 ± 6.6^{a}	129.63 ± 1.8^{a}	76.41 ± 0.9^{b}	60.57 ± 1.1^{c}	51.62 ± 0.2^{a}	51.55 ± 1.3^{a}	44.27 ± 1.0^{b}	2.88 ± 0.1^{b}	5.13 ± 0.09^{a}	5.02 ± 0.2^{a}
A. bisulcatus	**cotyledon**	108.23 ± 2.4^{a}	105.80 ± 3.4^{a}	95.47 ± 0.4^{b}	73.97 ± 0.7^{a}	74.98 ± 0.6^{a}	73.58 ± 0.7^{a}	102.88 ± 4.9^{a}	107.86 ± 0.6^{a}	99.92 ± 0.2^{a}	46.26 ± 1.9^{a}	47.77 ± 0.2^{a}	43.53 ± 0.5^{b}	1.63 ± 0.4^{a}	2.13 ± 0.2^{a}	1.94 ± 0.2^{a}
	root	963.60 ± 25.7^{b}	1234.00 ± 14.7^{a}	811.70 ± 5.0^{c}	336.00 ± 5.2^{a}	344.62 ± 5.0^{a}	295.28 ± 0.4^{b}	147.3 ± 2.3^{a}	90.08 ± 5.6^{b}	96.65 ± 1.8^{b}	47.35 ± 4.7^{a}	31.68 ± 2.1^{b}	40.63 ± 1.5^{a}	3.08 ± 0.02^{a}	2.24 ± 0.09^{b}	2.21 ± 0.1^{b}

FIGURE LEGENDS

Fig 1 Concentration of selenium in the root system (A) and in the cotyledons (B) of 14-days-old *A. membranaceus* and *A. bisulcatus* treated with 0 (control), 50 or 100 μM sodium selenate for 14 days. Different letters indicate significant differences according to Duncan-test (n=3, P≤0.05).

Fig 2 (A) Germination percentage of *Astragalus* species on agar media supplemented with 0 (control), 50 or 100 μM sodium selenate. Shoot (B) and root (C) fresh weight of 14-days-old *A. membranaceus* and *A. bisulcatus* plants treated with 0 (control), 50 or 100 μM selenate. Different letters indicate significant differences according to Duncan-test (n=15, P≤0.05). (D) Representative images showing 14-days-old *A. membranaceus* and *A. bisulcatus* plants grown on control or 50 or 100 μM selenate-containing agar media. Photographs show three representative individuals per treatment. Bars=3 cm.

Fig 3 (A) Selenium tolerance indexes (%) of *Astragalus* species treated with 50 or 100 μM selenate for 14 days. The 100% tolerance index of untreated plants is indicated by dashed line. Different letters indicate significant differences according to Duncan-test (n=10, P≤0.05). (B) Viability of primary root meristem cells in control and selenate-treated *Astragalus* species. Significant differences were determined by Student t-test and indicated by asterisks (n=15, *P≤0.05, **P≤0.01, ***P≤0.001, n.s.=non-significant). (C) Representative microscopic images indicating root tips of control (C) and selenate-treated *Astragalus* species stained with fluorescein diacetate. Bars=500 μm.

Fig 4 Root diameter (A), the thickness of the cortex (B) and the diameter of the stele in the roots (C) of control (Cont) and 50 or 100 μM selenate-treated (50 Se and 100 Se) *Astragalus* species after 14 days. The values of aniline blue (AB) fluorescence (pixel intensity) which refers to callose deposition are given in Control% (D). Auramine O staining of the control and Se-treated root sections of both species (E). Strong fluorescence can be seen at the xylem vessels (white arrows) and the endodermis and/or the exodermis (red arrows). Bar = 100 μm. Different letters refer to significant differences among the treatments within the same species according to Kruskal-Wallis ANOVA at $p<0.05$ (n= 6). Significant differences between the species within the same treatment were determined by Mann-Whitney U-test and are signified with asterisks (*$p<0.05$; **$p<0.01$, ***$p<0.001$, ns= non- significant).

Fig 5 (A) The level of superoxide in the root tips of *A. membranaceus* and *A. bisulcatus* treated with 0, 50 or 100 μM selenate for 14 days. Different letters indicate significant differences according to Duncan-test (n=10, $P\leq0.05$). (B) Representative fluorescent microscopic images showing DHE-stained root tips of *Astragalus* species. Bars=500 μm. (C) Representative photographs taken from NBT-stained cotyledons of control (0 Se), 50 or 100 μM selenate-treated *A. membranaceus* and *A. bisulcatus*. The blue discoloration refers to superoxide accumulation. Bar=1 cm. (D) Native-PAGE (10%) separation of NOX isoenzymes in cotyledon and root of *Astragalus* species treated with 0, 50 or 100 μM selenate for 14 days. The most representative protein band is indicated as “main band”. Additional putative isoenzymes are indicated by black arrows and newly appeared NOX isoenzymes are labelled by asterisks. (E) Total activity of SOD enzymes in the organs of *Astragalus* species supplemented with (50, 100

μM) or without (0 μM) selenate. Different letters indicate significant differences according to Duncan-test (n=3, P≤0.05). (F) Native-PAGE separation (10%) of SOD isoenzymes in cotyledon and root of control and selenate-treated *Astragalus* species.

Fig 6 The level of nitric oxide (A-D) and peroxynitrite (E-H) in intact root tips (A,B,E,F) and cotyledon cross-sections (C,D,G,H) of control (0 μM), 50 μM or 100 μM selenate-treated *A. membranaceus* and *A. bisulcatus*. Bars=500 μm. (I-L) Immunofluorescent detection of GSNO in cross-sections of roots (I and J) and cotyledons (K and L). Bars= 200 μm. Different letters indicate significant differences according to Duncan-test (n=5-6, P≤0.05). (M) Native-PAGE (6%) of *Astragalus* cotyledon and root extracts and staining for GSNOR activity. *A. membranaceus* and *A. bisulcatus* were treated with 0, 50 or 100 μM selenate for 14 days.

Fig 7 The intensity of 3-nitrotyrosine-related fluorescence in root (A) or cotyledon (C) cross sections of control and selenate-treated *A. membranaceus* and *A. bisulcatus*. Different letters indicate significant differences according to Duncan-test (n=5-6, P≤0.05). Representative fluorescent microscopic images showing cross sections of roots (B) and cotyledons (D) of *Astragalus* species treated with 0, 50 or 100 μM selenate for 14 days. Bars=200 or 500 μm.

Fig 8 Protein and tyrosine nitration pattern in cotyledon and root of control and selenate-treated *Astragalus* species (25 μg per lane). Silver-stained SDS gels (12%) and Western blots probed with a rabbit anti-nitrotyrosine polyclonal antibody (1:2000). Commercial nitrated BSA (NO_2-BSA)

was used as a positive control and molecule marker is shown as a protein weight indicator. Grey arrows indicate intensification in nitration, and white arrows show protein bands with decreased nitration. Selenate-induced, newly appeared protein bands are indicated by black arrows.

Fig 9 Schematic model summarizing the data obtained by this study. In the sensitive species, selenium exposure induces intense modification of root cell wall structure, disturbs microelement homeostasis and induces NO, superoxide and peroxynitrite accumulation as well as protein tyrosine nitration (nitro-oxidative stress). The observed alterations together lead to selenium-induced damages. In contrast, Se tolerant species shows slight cell wall modifications and non-disturbed microelement homeostasis. Additionally, selenium does not trigger NO, superoxide, peroxynitrite or 3-nitro-tyrosine formation, instead the high amount of endogenous NO storage (GSNO) and large nitroproteome decreases without the accumulation of NO suggesting that GSNO (or the nitrosoproteome) and the nitroproteome are able to buffer the amount of NO radical. In the hyperaccumulator, slight Se-triggered damages or the complete lack of damages can be observed. See details in the text. Abbreviations: 3NT=3-nitro-tyrosine.